

Reassembled Biosynthetic Pathway for a Large-scale Synthesis of CMP-Neu5Ac

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Received: 10 October 2003 / Accepted: 13 November 2003 / Published: 26 November 2003

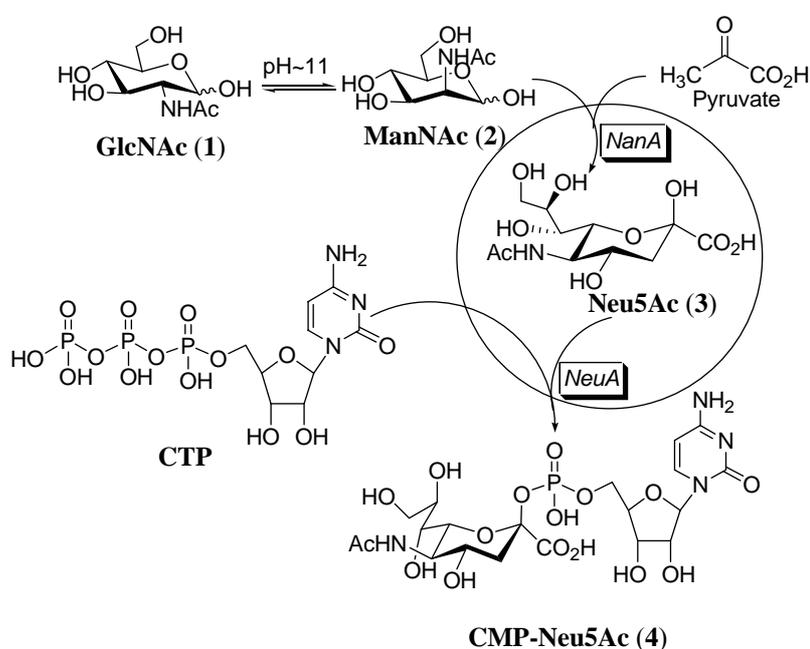
Abstract: CMP-Neu5Ac is an important sugar nucleotide for biosynthesis of sialic acid and its conjugates. In this paper, a large-scale production system of CMP-Neu5Ac by a single strain is reported. The co-expression of Neu5Ac aldolase (EC4.1.3.3) and CMP-Neu5Ac synthetase (EC 2.7.7.43) was achieved by constructing individual genes into one plasmid and having a single culture that has both NeuAc aldolase and CMP-Neu5Ac synthetase activities. Overall this system only employed *N*-acetylmannosamine, excess of pyruvate and CTP to produce CMP-Neu5Ac. This work has demonstrated that a large-scale synthesis of sialic acid-derived oligosaccharides could be achieved economically and efficiently through a single, biosynthetic pathway engineered microorganism.

Keywords: biosynthesis, cytidine 5'-monophospho-*N*-acetylneuraminic acid, NeuAc aldolase, CMP-NeuAc synthetase.

Introduction

It is well known that sialic acids (*N*-acetyl neuraminic acid) position within glycoconjugates, and it has become increasingly apparent that sialic acids are intimately involved in a number of important physiological phenomena and disease states, and they play essential roles in numerous biological processes such as cell recognition, viral infection, and toxin binding [1-3]. Generally, CMP-Neu5Ac (cytidine 5'-monophospho-*N*-acetyl neuraminic acid), the activated form of Neu5Ac, serves as the common activated sugar donor for all the sialyltransferases to transfer a single sialic acid to an acceptor or a growing oligosaccharide chain [4,5]. Thus, there is an increasing demand for its large scale production [6].

Figure 1. Synthesis of CMP-Neu5Ac in the whole cell reaction.

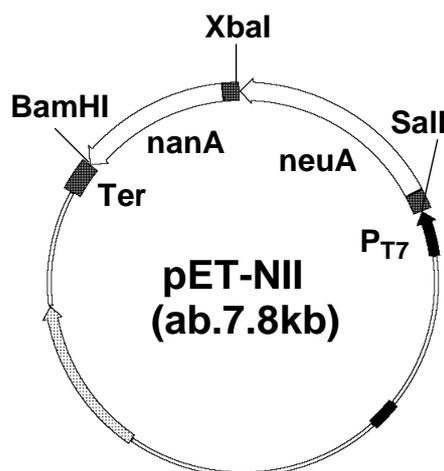


The synthesis of CMP-Neu5Ac is primarily catalyzed by the CMP-Neu5Ac synthetase, an enzyme that uses CTP (cytidine triphosphate) as energy source to condense with sialic acid and give rise to the activated form of Neu5Ac (Figure 1.). Neu5Ac aldolase (EC4.1.3.3) catalyzes the reversible cleavage of sialic acid to pyruvate and ManNAc [7]. CMP-Neu5Ac synthetase (EC 2.7.7.43) catalyzes the formation of CMP-Neu5Ac from Neu5Ac and CTP [8]. In the past, most of the enzymes used were from animal tissues, which contain only low levels of the synthetase activity and this made it difficult to provide CMP-Neu5Ac in large quantities with high quality. The first microbial enzyme used for the preparation of activated NeuAc was the CMP-Neu5Ac synthetase from *Neisseria meningitides*, but the specific activity of the synthetase in the cell extract was rather low [9,10]. More recently, the genes for

CMP-Neu5Ac synthetase from *E. coli* strains were cloned and overexpressed in *E. coli* [11-13]. Synthesis of CMP-Neu5Ac through bacterial coupling has been previously reported [6]. Inspired by the efficient and cost effective approach in the production of sugar nucleotides in the whole cell reaction developed by Kyowa Hakko, our group has recently developed a “superbug” strategy to produce oligosaccharides in large quantities [14,15]. The “superbug” reaction can be highly productive and cost effective through conventional fermentation by using cheap starting materials such as monosaccharides.

As reported herein, we assembled the genes of these two enzymes (*NanA*, *NeuA*) to form an artificial gene cluster onto a single plasmid (Figure 2.). Subsequent transformation of the plasmid into an *E. coli* strain affords a carbohydrate-producing bacterial strain. Such a strain can be fermented in large quantities and used for the synthesis of the sialylated oligosaccharides from inexpensive starting materials.

Figure 2. Plasmid map of CMP-Neu5Ac producing superbug. The map of superbug harboring two genes encoding enzymes involved in the biosynthetic pathway. Introduced restriction sites: Sall, XbaI, and BamHI.



Results and Discussion

Co-expression and characterization of recombinant enzymes.

The Neu5Ac aldolase gene (*nanA*) and CMP-Neu5Ac synthetase gene (*neuA*) were amplified from the chromosomal DNA of *E. coli* K12 and K1, respectively. The two DNA fragments obtained were cloned tandemly downstream of the T7 promoter, each with its own ribosome-binding site. The recombinant plasmid was used to transform *E. coli* BL21 (DE3) and the transformant was used for overexpressing NanA and NeuA. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) indicated that recombinant enzymes were overexpressed in the host strain with the target protein representing more than 70% of the total soluble protein and the coexpression had no adverse

effect on the levels of soluble protein (data not shown). Neu5Ac aldolase (EC4.1.3.3) from *E. coli* is stable between pH 6.0 to 9.0 with pH optimum around 7.7, thermostable up to 60°C. The gene of CMP-Neu5Ac synthetase (EC 2.7.7.43) has been cloned from *E. coli* K1 [10], *Neisseria meningitidis* [11], *Haemophilus ducreyi* [12] as well as murine pituitary cell [8]. The enzyme purified from *E. coli* K1 has a requirement for Mg²⁺ or Mn²⁺, and exhibits optimal activity between pH 9.0 and 10 in the presence of 30 mM MgCl₂, or is the most active near pH 7.5 in the presence of 5 mM MnCl₂. The apparent K_m for CTP and NeuAc are 0.31 mM and 4 mM, respectively. After induction, the enzyme activity of NeuA aldolase was obtained at a level of 4000 units/L and that of CMP-NeuAc synthetase was at a level of 200 units/L. The activities of the co-expression are comparable to those of the individual enzymes.

Isomerization of GlcNAc to ManNAc

The expensive ManNAc can be prepared practically from GlcNAc by epimerization of the *N*-acetylamino group at C-2. The epimerization can be achieved by using GlcNAc 2-epimerase (EC 5.1.3.8) and a continuous process for synthesis of NeuAc can be established by immobilizing enzymes in the membrane reactor [17]. Epimerase enzyme, however, is not easily available, which limits its application to large-scale production of NeuAc. Preparation of ManNAc from GlcNAc can also be accomplished by alkaline epimerization [18,19]. A number of processes have been described which provide isomerization of GlcNAc to ManNAc on a multigram scale using sodium hydroxide. Although GlcNAc dominates in the equilibrium mixture (the ratio of ManNAc to GlcNAc is approximately 1/4 ~ 1/3), ManNAc could be extracted into hot-ethanol to yield a ManNAc-enriched solution (about 80% to 88% pure). Such crude ManNAc can be used directly in the aldolase reaction, and the recovered GlcNAc could be recycled. In the first stage, epimerization of GlcNAc to ManNAc was achieved with an aqueous base at about pH~11. The equilibrium was reached after the mixture was allowed to stand for 2 days. To avoid using a large amount of hot ethanol due to the modest solubility of ManNAc, extraction with methanol was used to effectively enrich the ManNAc mixture (Table 1) [20].

Table 1. Solubility of GlcNAc and ManNAc in alcohols at 20 °C.

Alcohols	Solubility (g/L)		ManNAc/GlcNAc
	ManNAc	GlcNAc	
Methanol	90.6	4.2	21.6
Ethanol	8.0	0.7	11.4
<i>n</i> -Propanol	1.1	0.2	5.5
<i>i</i> -Propanol	1.0	0.2	5.0

After evaporation, the dry ManNAc/GlcNAc mixture was directly added to the enzyme reaction buffer. Typically ManNAc can be enriched in the range of about 75-80% in the mixture. The procedure was repeated using recovered GlcNAc to provide total 11.24 g of a crude solid mixture containing ~75% of ManNAc, and the filtrate was concentrated under reduced pressure to provide a ManNAc-enriched mixture (GlcNAc/ ManNAc = 1:4), according to analysis by $^1\text{H-NMR}$ spectroscopy (data not shown).

Synthesis of CMP-Neu5Ac in the whole cell reaction

The synthesis of CMP-Neu5Ac from ManNAc, pyruvate and CTP with the fusion protein was performed in the whole cell reactions at pH 7.5, in the presence of 10 mM MnCl_2 . The formation of the sugar nucleotide was monitored by TLC (eluant: *n*-BuOH/AcOH/ H_2O = 5:3:2) as well as by HPLC with UV detection (Figure 3A). Time course studies, as shown in Figure 4, indicated that the accumulation of CMP-Neu5Ac reached its peak at around 12 h in the whole cell reaction catalyzed by the fusion protein that has both NeuAc aldolase and CMP-Neu5Ac synthetase activities, and it was accumulated to 13.22 mM (8.12 g/L) in the reaction mixture. When it was exposed in the reaction mixture after longer time, however, CMP-Neu5Ac seemed to be decomposed since the concentration decreased.

Figure 3. CMP-Neu5Ac identification by HPLC. The reaction mixture of CMP-Neu5Ac synthesized in whole cell reaction was collected and analyzed by HPLC (A), and the purified CMP-Neu5Ac was collected and analyzed by HPLC (B).

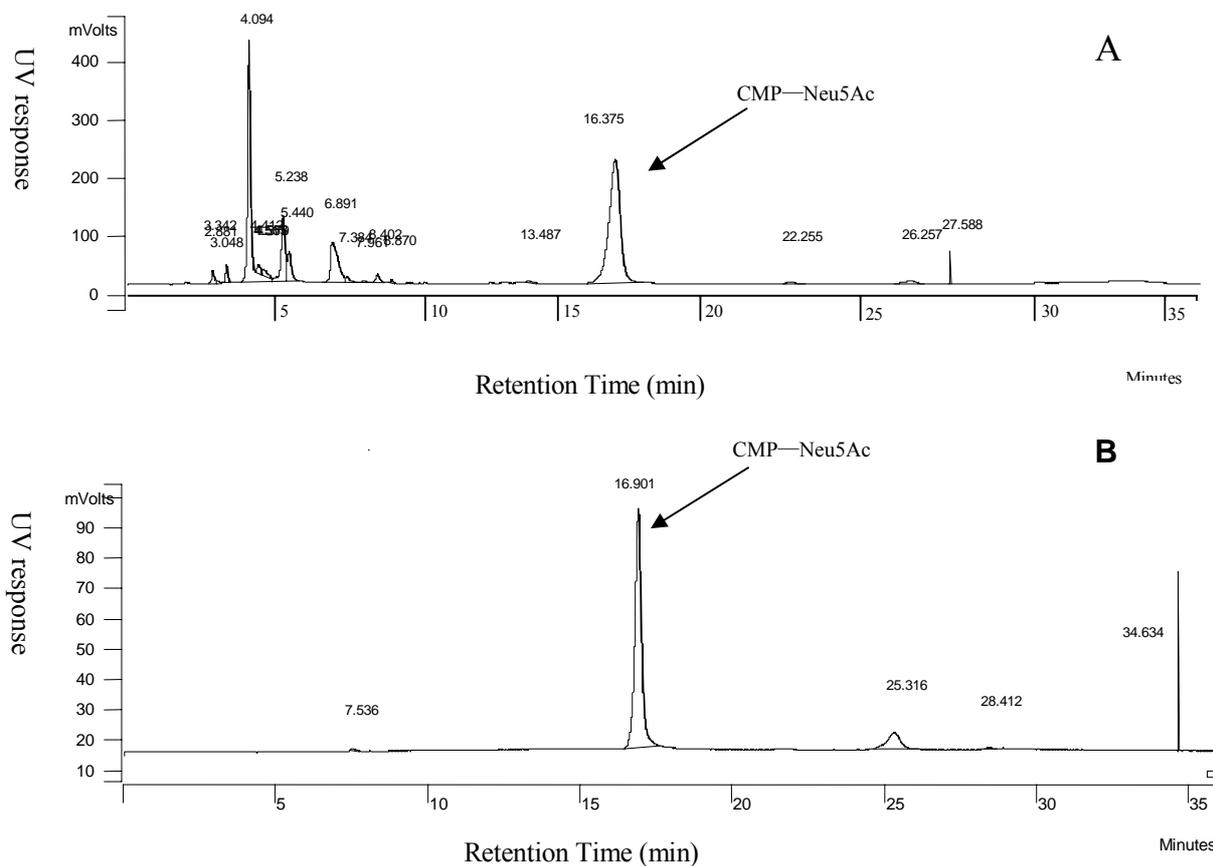
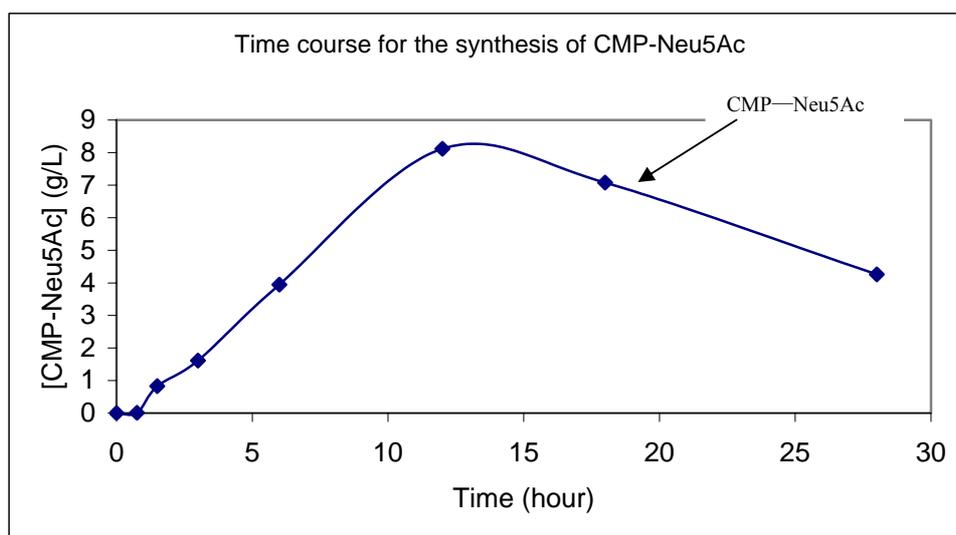


Figure 4. Time course for CMP-Neu5Ac production at a 2-mL scale. The accumulation of CMP-Neu5Ac reached its peak at around 12 h in the whole cell reaction catalyzed by the superbug containing fusion protein of both Neu5Ac aldolase and CMP-Neu5Ac synthetase. It was accumulated to 13.22 mM (8.12 g/L) in the reaction mixture. When it was exposed in the reaction mixture after longer time, however, CMP-Neu5Ac might be decomposed since the concentration decreased.



Analysis of CMP-Neu5Ac

CMP-Neu5Ac was analyzed by means of high-performance liquid chromatography with UV/Vis detection (Varian Prostar 320) using a Varian system equipped with a Microsorb 100 -NH₂ column, (250 × 4.6 mm). The concentration was measured at a flow rate of 0.75 mL/min, elution was performed with a linear gradient of 20 mM phosphate buffer (pH 6.2, composed of 16.16 mL of 1 M KH₂PO₄ and 3.84 mL of 1M K₂HPO₄) at increasing concentration from 6 mM to 20 mM, and of acetonitrile at decreasing concentration from 70% to 0% v/v, in a time period of 30 min, at injection volume of 20 μL, and UV detection at 272 nm.

Purification of CMP-Neu5Ac

Purification of the CMP-Neu5Ac from CTP, CMP, pyruvate, phosphate, inorganic salts and the mass of the bacteria cells was conducted by ion-exchange chromatography using the formate form of anion-exchange resin (Dowex 1×8) [21]. The column was eluted with a gradient of aqueous ammonium bicarbonate. The use of ammonium bicarbonate prevented hydrolysis of the extremely acid-labile CMP-Neu5Ac and provided it as the ammonium salt. Excess ammonium bicarbonate was easily removed by passing gel filtration column of the Bio-Gel P-2. Fractions containing CMP-

Neu5Ac were identified by TLC analysis and collected. The purity of the pooled collections was analyzed by HPLC (Figure 3B) and the final product was dried by lyophilization.

Conclusions

Biocatalytic approaches in which isolated enzymes, especially Leloir glycosyltransferases, are used are powerful and complementary alternatives to chemical synthesis of carbohydrates and glycoconjugates [22,23]. So far, a large number of mammalian glycosyltransferases have been cloned and employed in oligosaccharide synthesis; these enzymes include bovine α 1,3-galactosyltransferase [24] and human α 2,3-sialyltransferase [25] and α 1,3-fucosyltransferase [26]. However, the utility of these enzymes in large-scale synthesis of glycoconjugates is partially limited by the high cost of eukaryotic cell culture and by the low level of protein expression. Most bacterial glycosyltransferases, on the other hand, are easily expressed at high levels in cheaper prokaryotic expression systems. Moreover, bacterial glycosyltransferases seem to have broader acceptor substrate specificities, thereby offering a tremendous advantage over mammalian enzymes in chemoenzymatic synthesis of oligosaccharides and their derivatives. Nowadays availability of more and more new glycosyltransferases has increased the demand for sugar nucleotides in the production of oligosaccharides and glycoconjugates. Enzymatic synthesis of sugar nucleotides is more attractive than chemical methods because the yields are high and no organic solvents are used. CMP-Neu5Ac is synthesized in vivo by reaction of CTP and sialic acid in the presence of CMP-Neu5Ac synthetase. Endo et al has reported a large-scale production system of CMP-Neu5Ac by two recombinant *E.Coli* strains that overexpressed the genes of CMP-Neu5Ac synthetase and CTP synthetase and *corynebacterium .ammoniagenes* using Neu5Ac, orotic acid and fructose as starting materials [6]. Our approach here is the coexpression of two enzymes involved in the CMP-Neu5Ac biosynthetic pathway in one microbial strain to modify the host cells towards the accumulation of the desired product. Compared to the Kyowa Hakko technology which involves several plasmids and multiple bacterial strains, our approach achieves the same goal with the use of only one plasmid and a single strain. All the enzymes essential for CMP-Neu5Ac are in one *E.Coli* strain. CMP-Neu5Ac can be produced on a large-scale through an artificial bacterial strain which has both Neu5Ac aldolase and CMP-Neu5Ac synthetase activities by using CTP, sodium pyruvate and ManNAc as starting materials. This artificial cluster, consisting of only two genes, together with inexpensive starting material GlcNAc make this approach more attractive. The simplicity of the synthetic cycle, and thus that of the plasmid, allows for further modification, such as the addition or replacement of genes. Work is in progress to incorporate a α 2,3-sialyltransferase gene into pET-NII to form plasmids consisting of three genes for the synthesis of sialylated oligosaccharides starting from N-acetylglucosamine.

In summary, this paper describes the integrated, efficient and economical system for the large-scale production of CMP-Neu5Ac through introducing an artificial gene cluster into a single *E.coli* strain. It

Co-expression and purification of Neu5Ac aldolase gene and CMP-Neu5Ac synthetase

The recombinant plasmid pET-NII containing both nanA gene and neuA gene was transformed into *E. coli* BL21 (DE3) and the transformant was grown in LB (50 mL) with ampicillin ($150 \mu\text{g mL}^{-1}$) overnight (30°C , 225 rpm), then transferred into freshly prepared LB (1 L) with ampicillin ($150 \mu\text{g mL}^{-1}$) for another 3 h at 37°C . When OD_{600} reached 0.8-1.0, the culture was induced with $400 \mu\text{M}$ isopropyl-1-thio- β -D-galactopyranoside (IPTG) for 4 h at 37°C . The cells were harvested by centrifugation (4500 rpm, 10 min, 4°C), disrupted by sonication on ice in 25 mL binding buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole), clarified by centrifugation and loaded onto a Ni-NTA column (Qiagen). The column was first washed with binding buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole), then with washing buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 20 mM imidazole), followed by elution with elution buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 200 mM imidazole). The eluants were concentrated by ultrafiltration, dialyzed against dialysis buffer (20% glycerol, 20 mM, Tris-HCl, pH 7.9).

Isomerization of N-Acetylglucosamine to N-Acetylmannosamine

The isomerization of *N*-acetylglucosamine was carried out using 0.055 M of NaOH at room temperature. Typically, 20 g of GlcNAc was dissolved in 200 mL of NaOH aqueous solution (0.055M) and allowed to stand at room temperature for 48 h. The resulting pale yellow solution was neutralized by addition of glacial acetic acid and evaporated to dryness under high vacuum. The residue was extracted by refluxing it in 100 ml methanol for 1 h and then standing at room temperature for 4 h, and filtered to remove GlcNAc. The ratio of ManNAc to GlcNAc was determined by $^1\text{H-NMR}$ analysis of the dry mixture, particularly by looking at the integrals of both anomeric protons in α - and β - isomers.

Synthesis of CMP-NeuAc in whole cell reaction

The synthesis of CMP-Neu5Ac was carried out in a 200 mL flask containing 50 mL of reaction mixture. Cell pellets (2.5 g, wet weight) suspended in a Tris-HCl (50 mM, pH 7.5) were added to a reaction mixture of ManNAc-enriched solid mixture (GlcNAc/ManNAc, 3/1) (370 mg, 1.25 mM), sodium pyruvate (330 mg, 3.0 mM), CTP (480mg, 1.0 mM), MnCl_2 (30 mM), DTT (10 mM) in Tris-HCl buffer (50 mM, pH 7.5). The total volume was brought up to 50 mL by addition of water. The reaction was incubated in an isotherm incubator with agitation in 400 rpm at 37°C for 12 h, when TLC analysis [*n*-BuOH-AcOH- H_2O = 5:3:2 (vol/vol/vol)] indicated that reaction was complete. The reaction was quenched by addition of 50 mL 95% ethanol. The cells were separated from the reaction mixture by centrifugation and washed with deionized water. The combined supernatants were centrifuged again to further remove the pellets. After concentration, the reaction mixture was purified by a Bio-Rad P-2 gel filtration column with distilled water as the mobile phase. The isolation process

was monitored by UV (at 272 nm) and TLC analysis analysis [*n*-BuOH-AcOH-H₂O = 5:3:2 (vol/vol/vol)].

Purification of CMP-Neu5Ac

The reaction mixture was centrifuged at 15,000 rpm for 30 min at 4°C to remove the proteins. The pellet was resuspended in 50% aqueous alcohol solution and centrifuged again. The wash step was repeated three times. The combined supernatants were concentrated to a small volume and purified by ion-exchange chromatography. The anion resin (Dowex 1×8) was pretreated with 50 mM of aqueous sodium formate solution to load the column (25 × 500 mm) with formate form. The column was first washed with two volumes of distilled water to remove the monosaccharides and metal ions, and then one volume of 50 mM ammonium bicarbonate solution was used as eluant followed by a 50 mM gradient of ammonium bicarbonate. The fractions containing CMP-Neu5Ac, as evidenced by TLC analysis (eluant: *n*-BuOH-AcOH-H₂O = 5:3:2), were pooled and concentrated to give a white residue. Excess ammonium bicarbonate was removed by dissolving the residue in small amount of water and passing through a column (2.5 × 125 cm) of Sephadex G-15 (Sigma), which has been equilibrated and eluted with distilled water to remove the contamination salts before use. The fractions containing the CMP-Neu5Ac, as identified from TLC analysis were pooled and lyophilized to give rise to the final product. Components other than CMP-Neu5Ac were further removed by means of gel-filtration (size exclusion chromatography) with a column of Bio-Gel P-2 (1.5 × 80 cm) eluting with distilled water. Fractions containing CMP-Neu5Ac were collected, freeze-dried to afford 365 mg of CMP-Neu5Ac (57% based on CTP). The ¹H-NMR (D₂O) of CMP-Neu5Ac was recorded and it was in good accordance with the literature [21]: δ 8.15 (s, 1H, -NHAc), 7.87 (d, 1H, *J* = 7.3 Hz, H-6 of cytidine), 6.25 (d, 1H, *J* = 8.1 Hz, H-5 of cytidine), 5.91 (d, 1H, *J* = 4.0 Hz, H-1 of ribose), 4.26 (t, 1H, *J* = 4.0 Hz, H-2 of ribose), 4.23 (t, 1H, *J* = 4.9 Hz, H-3 of ribose), 4.17 (m, 3H, H-5a, 5b, H-4 of ribose), 4.09~3.98 (m, 2H, H-9a and H-8), 3.90~3.80 (m, 3H, H-5, H-6, H-4), 3.59~3.53 (dd, 1H, *J* = 11.4, 6.5 Hz, H-9b), 3.40 (d, 1H, *J* = 9.7 Hz, H-7), 2.42 (dd, 1H, *J* = 12.9, 4.9 Hz, H-3eq.), 1.98 (s, 3H, -CH₃), 1.64~1.54 (ddd, 1H, *J* = 12.2, 11.2, 4.7 Hz, H-3ax.); ¹³C-NMR (D₂O): δ 174.89 (-C=O), 174.60 (C-1 of NeuAc), 166.31 (C-2 of cytidine), 157.97 (C-4 of cytidine), 141.73 (C-5 of cytidine), 100.24 (C-6 of cytidine), 96.81 (C-2 of NeuAc), 89.25 (C-1 of ribose), 83.09 (C-4 of ribose), 74.43 (C-2 of ribose), 71.95 (C-3 of ribose), 69.75 (C-5 of ribose), 69.24 (C-6 of NeuAc), 68.97 (C-8 of NeuAc), 67.00 (C-4 of NeuAc), 65.10 (C-7 of NeuAc), 63.09 (C-9 of NeuAc), 51.97 (C-5 of NeuAc), 41.27 (C-3 of NeuAc), 22.29 (-CH₃). MS (ESI): 634.91 (M + Na⁺); 656.90 (M + 2Na⁺).

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Sample Availability: Available from the authors.